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Genetic evidence for activation of the positive transcriptional regulator Xy1R, a member of the NtrC family of regulators, by effector binding
A Delgado and JL Ramos

2) J Bacteriol 1992 Feb;174(3):711-24
Nucleotide sequence and functional analysis of the complete phenol/3,4-dimethylphenol catabolic pathway of Pseudomonas sp. strain CF600.
Shingler V, Powlowski J, Marklund U.

Thank you,
David Steadman

Genetic Evidence for Activation of the Positive Transcriptional Regulator XylR, a Member of the NtrC Family of Regulators, by Effector Binding*

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The XylR protein positively controls expression from the *Pseudomonas putida* TOL plasmid σ^{54} -dependent Pu and Ps promoters, in response to the presence of aromatic effectors such as *m*-xylene, *m*-methylbenzyl alcohol, and *p*-chlorobenzaldehyde in the culture medium. XylR also autoregulates its own synthesis. A mutant XylR regulator called XylR7 was isolated after nitrosoguanidine mutagenesis of the wild-type gene and phenotypic selection for mutants that had acquired the ability to recognize *m*-nitrotoluene, a nitroarene that is not an effector for the wild-type regulator. The mutant regulator exhibited a single point mutation that resulted in a change in codon 172 (GAA \rightarrow AAA), which should result in a Glu \rightarrow Lys change in the polypeptide chain. The effector profile of the mutant regulator was determined by measuring β -galactosidase from a fusion of the Pu promoter to a promoterless *lacZ* gene. The results showed that the mutant regulator had acquired the ability to recognize *m*-nitrotoluene, and retained the wild-type regulator's ability to recognize most of the wild-type effectors. Full transcriptional activation of the Pu promoter by XylR7, as with the wild-type XylR protein, requires its full modular structure, namely the σ^{54} recognition site, the integration host factor binding site, and the upstream activation sequences. The XylR7 regulator did not stimulate transcription from the Ps promoter in response to the presence of its effectors, and autoregulated its own synthesis at low levels.

The TOL plasmid pWW0 of *Pseudomonas putida* encodes the genetic information for the mineralization of toluene and xylenes via benzoate and toluates (1). The genes of the catabolic pathways are organized in four transcriptional units. Two of these, the "upper" and the *meta* pathway operons, contain the *xyl* structural genes encoding the corresponding catabolic enzymes for the oxidation of toluene/xylenes via benzoate/toluates to amphibolic intermediates, whereas the two remaining genes, *xylR* and *xylS*, encode regulatory proteins. Fig. 1 summarizes the transcriptional loops in cells growing on aromatic hydrocarbons.

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The XylR protein is the ultimate regulator involved in the transcriptional activation of the TOL catabolic pathways. The *xylR* gene is constitutively transcribed from two tandem promoters (2), but the XylR protein only stimulates transcription from its cognate regulated promoters, the upper pathway operon Pu, and the *xylS* gene promoter Ps, in response to the presence of a series of aromatics such as *m*-xylene, *m*-methylbenzyl alcohol, and *p*-chlorobenzaldehyde (3). Activation of transcription from Ps triggers a cascade system, the hyperproduction of the XylS protein in turn stimulating transcription from the *meta*-cleavage pathway operon to obtain the breakdown of the aromatics to amphibolic intermediates (4, 5).

The XylR-activated promoters on the *P. putida* TOL plasmid Ps and Pu belong to the σ^{54} -12/-24 family, and are dependent on the RpoN σ -factor (also called NtrA and σ^{54}). In addition, transcription from Pu but not from Ps is also dependent on IHF¹ (6, 7). As is the case with other σ^{54} -dependent promoters (8, 9), upstream activation sequences (UASs) located between 120 and 180 base pairs from the main transcription initiation point are necessary for XylR-mediated induction by toluene, xylenes, and other pathway substrates (6, 7, 10). Close proximity between the upstream bound activator and the promoter site is believed to be brought about by DNA loop formation, which is apparently aided in the case of the Pu promoter by the binding of the IHF protein at a site between the promoter and upstream activator sequence (6, 7).

The XylR protein shows significant sequence similarities with NtrC and other members of this family of regulators (11, 12). In the case of NtrC and other members of the family, activation of the regulator involves its phosphorylation mediated by a protein kinase that functions as the sensory element (13). The XylR regulator, both in *P. putida* and when cloned in *Escherichia coli*, activates transcription from Pu and Ps promoters in response to effectors. This has been taken as evidence of the activation of the regulator after effector binding. In this article we show that a mutant XylR exhibiting a single amino acid change at the N-terminal end acquires the ability to recognize *m*-nitrotoluene, which is not an effector for the wild-type protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Bacterial strains used in this work were *E. coli* MC4100 (F⁺, *araD139*, Δ *lactF-lacU169*), *rpsL150*, *relA*, *flb5301*, *ptsF25*, *deoC1*), *E. coli* ET8000 (*rbs*, *lacZ::IS1*, *gvrA*, *hutC*), and *E. coli* ET8045 (ET8000 [*trnA::Tn10*], Tet^r).

Plasmids used were pTS174 (Cm^r, *xylR*⁺, P15 replicon) (14), pAD1 (Cm^r, *xylR7*, P15 replicon) (this study), pRD579 (Ap^r, *Pu::lacZ*, pR1 replicon) (15), pAH100 (Ap^r, *Ps::lacZ*, pR1 replicon) (16), pAH120 (Ap^r, *Pr::lacZ*, pR1 replicon) (16), pTZ19 (Ap^r, cloning and sequencing vector)

¹ The abbreviations used are: IHF, integration host factor; UAS, upstream activation sequence.

Abril and Ramos (18) have reported that the Pu promoter exhibits a modular structure that is required for full transcrip-

TABLE I
Effector profile of XylR and XylR7 as determined by using a Pu::lacZ fusion

E. coli ET8000 (pRD579) also bearing either pTS174 (xylR) or pAD1 (xylR7) were grown with vigorous shaking in the absence or presence of 1 mM of the above aromatics dissolved in dimethylformamide. Other experimental details are given under "Experimental Procedures." The data are the averages and the standard deviations of at least three independent determinations.

Effector	β -Galactosidase	
	XylR	XylR7
	units	
None	300 \pm 15	150 \pm 20
m-Xylene	1550 \pm 240	500 \pm 70
m-Methylbenzyl alcohol	1150 \pm 170	450 \pm 50
p-Chlorobenzaldehyde	1750 \pm 250	720 \pm 10
o-Nitrotoluene	1350 \pm 220	1400 \pm 300
m-Nitrotoluene	150 \pm 30	950 \pm 150
p-Nitrotoluene	1150 \pm 150	850 \pm 60

tional stimulation, namely, the σ^{54} recognition site, the IHF binding site, and the UASs. The requirement for σ^{54} (NtrA) and IHF for transcriptional stimulation from Pu by XylR7 was demonstrated using appropriate *E. coli* mutant strains and their isogenic parental strains. In fact, stimulation of transcription by XylR7 from the wild-type Pu promoter was not observed in the NtrA⁻ strain *E. coli* ET8045, in contrast with full induction in ET8000 (Table II). As with the wild-type XylR regulator, induction from Pu with XylR7 and effectors in an IHF⁻ background was about 10–25% of the level in the IHF⁺ background. We also assayed activation by XylR and XylR7 from the wild-type Pu promoter in pERD401 and a mutant Pu derivative lacking the UAS1 region in pERD412. The mutant Pu promoter exhibited a deletion from -160 to -180 with respect to the main transcription initiation point (UAS1). With this mutant Pu promoter, no transcriptional activation in response to aromatic effectors was observed with either XylR or XylR7, suggesting that this UAS region is required for activation of transcription by XylR7 (not shown). Therefore, the mutation in the XylR regulator seems to affect primarily interactions with the effector, and requires the full modular structure of the Pu promoter to stimulate transcription.

Stimulation of Transcription from the xylS Gene Promoter by XylR and XylR7—To determine whether the mutant XylR7 regulator stimulated transcription from the Ps promoter, *E. coli* ET8000 bearing pAH100 (Ps::lacZ) was transformed with pTS174 and pAD1. We determined β -galactosidase activity in the absence and presence of 5 mM m-xylene, m-nitrotoluene, and m-methylbenzyl alcohol. The induction pattern from Ps by XylR was similar to that reported by Holtel *et al.* (16): β -galactosidase was observed in cells growing in the presence of m-xylene and m-methylbenzyl alcohol but not with m-nitrotoluene (Table III). Unexpectedly, XylR7 did not mediate activation of transcription from Ps in response to m-nitrotoluene or the other aromatics (Table III).

XylR7 Weakly Regulates Its Own Synthesis

The wild-type XylR protein autoregulates its own synthesis. The fusion pAH120 (Pr::lacZ) was used to study whether XylR7 also autoregulates its own synthesis; XylR was used as a control. As shown in Table IV, the transcriptional level from Pr in the presence of XylR with and without effector was reduced to about 40–50% of the level in the absence of the regulator. With the XylR7 regulator, the level of repression from the Pr promoter was only about 25% of the level in its absence. These results suggest that the XylR7 regulator showed an altered ability to regulate its own synthesis (Table IV).

TABLE II
Activation of the Pu promoter by XylR and XylR7 in different genetic backgrounds

The *E. coli* ET8000 and ET8045 were transformed with pRD579 (Pu::lacZ) and either pTS174 (xylR) or pAD1 (xylR7), and were grown in the presence or absence of 1 mM m-methylbenzyl alcohol (m-MBA) or m-nitrotoluene (m-NT). Other experimental details are given under "Experimental Procedures."

Strain	Relevant background	Effector	β -Galactosidase Pu::lacZ
ET8000	NtrA ⁺ , XylR ⁺	None	300
		m-MBA	1700
		m-NT	200
ET8045	NtrA ⁺ , XylR ⁺	None	20
		m-MBA	20
		m-NT	30
ET8000	NtrA ⁺ , XylR7	None	150
		m-MBA	550
		m-NT	800
ET8045	NtrA ⁺ , XylR7	None	30
		m-MBA	30
		m-NT	30

TABLE III
Activation of the Ps promoter by XylR and XylR7

E. coli ET8000 (pAH100) was transformed or not with pTS174 (xylR) and pAD1 (xylR7). Bacteria were grown for 16 h with vigorous shaking in the absence or presence of 5 mM m-xylene, m-methylbenzyl alcohol, and m-nitrotoluene. The data are the averages and standard deviations of at least three independent determinations. Other experimental details are given under "Experimental Procedures."

Effector	β -Galactosidase	
	XylR	XylR7
None	140 \pm 20	140 \pm 30
m-Xylene	360 \pm 20	170 \pm 20
m-Methylbenzyl alcohol	1000 \pm 150	240 \pm 35
m-Nitrotoluene	50 \pm 10	150 \pm 30

TABLE IV
Regulation of expression from Pr by XylR and XylR7

Conditions are as described in the legend to Table II except that *E. coli* bore pAH120 instead of pAH100. The data are the averages and standard deviations of at least three independent determinations. Number in parentheses are percentage of activity.

Effector	β -Galactosidase activity; regulator		
	None	XylR	XylR7
None	580 \pm 50 (100)	260 \pm 25 (46)	430 \pm 30 (75)
m-Methylbenzyl alcohol	520 \pm 40 (100)	200 \pm 20 (39)	380 \pm 40 (73)

DISCUSSION

The XylR protein, which stimulates transcription from the σ^{54} -dependent Pu and Ps promoters in the TOL plasmid pWW0 of *P. putida* (4, 25, 26), belongs to the NtrC family of regulators (12, 27). Within this family the greatest similarity, based on the algorithm of Needleman and Wunsch (22, 23), was to DmpR, the regulator for the phenol meta-cleavage pathway for phenol degradation by *P. putida* (28). The XylR protein also has a high similarity score with AlgB (29) and PilR (30) of *Pseudomonas aeruginosa*, DctD of *Rhizobium meliloti* (31), and FliD of *Caulobacter crescentus* (32), among the members of the NtrC family. It should be noted that the similarity scores of XylR with NifA from *Klebsiella pneumoniae* (33), AnFA from *Azotobacter vinelandii* (34), and TyrR from *E. coli* (35) (members of the NtrC family) were not significant, which probably reflects evolutionary divergences within the family.

Multiple alignment of the protein sequences of the NtrC family revealed the greatest similarity extending to the central region (residues 234–472 in XylR; see Fig. 2) (27), which is

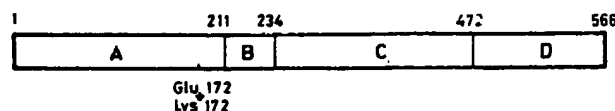


FIG. 2. Modular structure of the XylR regulator. The domains A, B, C, and D were established through multiple alignments of the highly homologous members of this family of regulators. Numbers indicate the boundaries of the domains in the XylR regulator, and Glu¹⁷² → Lys indicates the mutation in the XylR7 regulator.

probably involved in ATP hydrolysis, dimerization, and interaction with σ^{54} (27). The DNA binding motif at the C-terminal end, where a potential α -helix turn α -helix motif (residues 534–553 in XylR) was found, also seems to be conserved. The N-terminal region of XylR did not exhibit sequence conservation with the other members of the family, except for DmpR (28). This region has been proposed to be responsible for signal/sensor reception. In fact, the NtrC regulator, the prototype protein of this family, is activated by phosphorylation of an aspartyl residue at the N-terminal region by the kinase activity of NtrB, a protein that senses cellular N status (13, 36, 37). This mode of activation has been shown to work for the activation of other members of the family and other prokaryote transcriptional regulators, e.g. OmpR and NarL (38, 39). In contrast, activation of the XylR and DmpR regulators, which share 64% similarity at their N-terminal end, and are involved in the activation of catabolic pathways for metabolism of aromatics by *Pseudomonas*, seems to involve direct interaction of the effector and the regulator rather than phosphorylation. Evidence for this hypothesis arises from the following facts. (i) These regulators, when cloned in a host other than their natural host (e.g. *E. coli*) only promoted transcription from their cognate regulatable promoters in response to the presence of aromatic effectors (3, 28). (ii) In the case of XylR, the effector profile of the regulator has been studied in *P. putida*, its natural host, and *E. coli*; these studies included a number of aromatic hydrocarbons, benzyl alcohol, and benzaldehyde derivatives. The pattern of induction in *P. putida* and *E. coli* was similar, suggesting that the regulator response is specific (3). (iii) In this study we have shown that the substitution of Lys for Glu¹⁷² in the XylR regulator leads to a mutant regulator that acquires the ability to stimulate transcription in response to *m*-nitrotoluene, a character not present in the wild-type regulator. The fact that an alteration in the protein sequence leads to alterations in the profile effector suggests, but does not prove, that residue 172 is important for interaction(s) with the effector.

We found that stimulation of transcription from the Pu promoter by effector-activated XylR7 (as was also the case with the wild-type regulator) required the full modular structure of the Pu promoter. This suggests that the mechanism of XylR7 activation of transcription from the Pu promoter is similar to that of the wild-type regulator; therefore the main alteration in XylR7 seems to involve interactions with the effector. However, a surprising finding was that this mutant regulator was unable to promote transcription from Ps, and weakly autoregulated its own synthesis (Tables III and IV). These findings indicate that XylR7 may be affected in interactions with its target DNA sequences at the overlapping Ps/Pr promoters, which are similar but not identical to those in the Pu promoter. Differences in the activation of Pu and Ps has been noted before. (i) Full stimulation of transcription from Pu required IHF protein, whereas activation of the Ps promoter was IHF-independent (6, 7, 16), and (ii) stimulation of transcription by wild-type XylR activated by effectors from the Pu promoter was about 4-fold stronger than stimulation from the Ps promoter under all con-

ditions tested.² Because the hydropathic profile of the region around residue 172 is altered due to the substitution of a lysine residue for a glutamyl residue, the alteration induced by this amino acid change may have been transmitted to other regions of the regulator in the three-dimensional structure of the regulator (i.e. the DNA binding domain), leading to subtle alterations in contacts with the corresponding UASs. Interactions at the Ps/Pr promoters may thus have been impeded, whereas this probably did not occur with the Pu promoter.

In summary, XylR is homologous to other regulators of the NtrC family, which includes proteins from different genera; however, it differs from the prototype protein of this family in that its interconversion between active and inactive forms is likely to be mediated by effector binding rather than by covalent modification catalyzed by a sensory protein.

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² S. Marqués, A. Holtel, K. N. Timmis, and J. L. Ramos, submitted for publication.